REMARKS

The Office Action dated May 14, 2004 presents the examination of claims 2, 4, 6, 9, 14-19, and 21-24. No amendments are made herein, and no new matter is inserted into the application.

Interview

An interview was held with the Examiner at the United States Patent and Trademark Office on August 17, 2004. The Examiner's assistance in expediting prosecution of the present invention is very much appreciated. In the Interview Summary, the Examiner writes:

> Applicant argues that the cited references are not combinable, even if combined they would not teach the instant invention, and the invention produces unexpected results. Applicant will respond as such to the outstanding action for further consideration.

The prior art and the obviousness rejections of record were discussed in detail during the interview. In particular, Applicants' representative stressed that the skilled artisan would not combine the cited references, and even if the cited references were hypothetically combined, they still would not render the present invention obvious.

Applicants respectfully submit that the following remarks present on the record a full response to the issues discussed during the interview, and clearly demonstrate that the present invention, as defined in the claims, is not obvious over the cited references, either alone or in combination. Therefore, Applicants respectfully request that the Examiner withdraw all rejections of record, and issue a Notice of Allowance indicating that claims 2, 4, 6, 9, 14-19, and 21-24 are allowed.

Request for Interview

If, for any reason, entry of the instant Reply does not place the present application into condition for allowance, the Examiner is respectfully requested to contact Kristi L. Rupert, Ph.D. (Reg. No. 45,702) at the phone number of the undersigned below, to conduct a telephonic interview.

Rejections under 35 U.S.C. § 103(a)

Claims 2, 4, 6, 16, 17, 18, 22, and 23 are rejected under 35 U.S.C. § 103(a) for allegedly being obvious over Gorski et al. (*Clinical Chemistry*, 43(1):193-195, 1997) in view of Maatman et al. (*Biochem. J.* 288:285-290, 1992) and Simon et al. (*J. Biol. Chem.* 272(16):10652-10663, 1997).

Claim 9 is rejected under 35 U.S.C. § 103(a) for allegedly being obvious over Gorski et al. in view of Maatman et al. and Simon et al., and further in view of Kimura et al. (*J. Biol. Chem.*, 266(9):5963-5972, 1991).

Claims 19 and 21 are rejected under 35 U.S.C. § 103(a) for allegedly being obvious over Gorski et al. in view of Maatman et al. and Simon et al., and further in view of Galaske et al. (*Pflugers Archives Euro. J. Physiol.*, 375(3):269-277, 1978).

Claims 14 and 15 are rejected under 35 U.S.C. § 103(a) for allegedly being obvious over Gorski et al. in view of Maatman et al. and Simon et al., and further in view of Zuk et al. (United States Patent No. 4,281,061).

Applicants respectfully traverse the rejections under 35 U.S.C. § 103(a). Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

I. THE PRESENT INVENTION

The present invention is directed to a method for diagnosis or prognosis of kidney disease in humans by detecting liver-type fatty acid binding protein (L-FABP) contained in a specimen collected from a human. The cited references do not teach or even suggest such a method for diagnosis or prognosis of kidney disease.

II. THE CITED PRIMARY REFERENCES

Gorski et al. discloses that plasma FABP concentration is markedly increased in patients with chronic renal failure. However, the study by Gorski et al. focused specifically on heart-type FABP (H-FABP) as a marker for myocardial infarction. As such, the FABP studied by Gorski et al. is H-FABP rather than L-FABP.[1] Gorski et al. fails to teach or suggest the diagnosis or prognosis of kidney disease in humans by the detection of L-FABP.

Maatman et al. discloses the existence of L-FABP and H-FABP in human kidneys, and further discloses that L-FABP may participate in the renal excretion of metabolites and may inhibit nephrotoxicity by binding with drugs. However, Maatman et al. fails to teach or suggest the diagnosis or prognosis of kidney disease in humans by the detection of L-FABP.

Simon et al. utilizes L-FABP as a model for investigating the molecular mechanisms that regulate gene transcription within and between epithelial cells that line the intestine and the nephrons of the kidney. Simon et al. discloses that a specific DNA sequence (i.e., a heptad repeat) existing in the upstream region of the L-FABP gene forms stable complexes with nuclear proteins in gel mobility shift assays. However, Simon et al. fails to teach or

[1] The disclosure of H-FABP in Gorski et al. and the differences between H-FABP and L-FABP are discussed in detail in Appendix I, attached hereto.

suggest the diagnosis or prognosis of kidney disease in humans by the detection of L-FABP.

III. BASIC REQUIREMENTS OF A PRIMA FACIE CASE OF OBVIOUSNESS

In order to establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation for the skilled artisan to combine reference teachings. Second, there must be a reasonable expectation of success. Third, the prior art references, once combined, must teach or suggest all of the claim limitations. U.S. Pat. & Trademark Off., Manual Pat. Examining Proc. § 2142 (8th ed. Rev. 2 2004). The suggestion or motivation to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. In re Vaeck, 947 F.2d 488 (Fed. Cir. 1991).

In the instant case, the Examiner has failed to make a *prima facie* case of obviousness. As discussed in detail below, the skilled artisan would not be motivated to combine the cited reference, nor would there be any reasonable expectation of success. Finally, even if all of the references were hypothetically combined, the combination thereof would still fail to teach all of the claim limitations of the instant claims.

IV. LACK OF MOTIVATION TO COMBINE GORSKI ET AL., MAATMAN ET AL., AND SIMON ET AL.

IV(A). THE EXAMINER'S RATIONALE TO COMBINE IS IMPROPER

As rationale for combining the Gorski et al., Maatman et al., and Simon et al. references, the Examiner writes,

> It would have been obvious to one of ordinary skill in the art at the time the invention was made to use the liver-type fatty acid binding protein as taught by Maatman et al., having proven function in the kidney (nephron) as taught by Simon et al. to detect the specific kidney diseases relating to FABP in the method of Gorski et al. because Maatman et al. taught that "the liver-type FABP binds various ligands and may be involved in the renal excretion of exogenous and endogenous nephrotoxicity." (See, page 4 of the Office Action).

Applicants respectfully disagree that the skilled artisan would be motivated to combine the references as stated by the Examiner. In this regard, the Examiner's reliance on the quoted statement above by Maatman et al. to provide the requisite motivation is improper. The comments by Maatman et al., when not taken in isolation, only provide mere speculation as to the physiological relevance of L-FABP. Specifically, Maatman et al. states,

> We can only speculate on the physiological relevance of the two FABP types in kidneys. The liver-type FABP binds various ligands and may be involved in the renal

> excretion of exogenous and endogenous metabolites. The liver-type FABP also binds some drugs [2,3], and may in this way prevent nephrotoxicity.

See, page 289, right column, lines 4-9.

From the above passage, it is clear that Maatman et al. speculates that L-FAPB may prevent nephrotoxicity by binding to drugs and causing their excretion from the liver. This speculation does not in any way suggest that there is a correlation between the levels of L-FABP in a human specimen and the diagnosis of kidney diseases. In other words, a possible function for L-FABP in the kidney does not shed light on the normal or abnormal levels of FABP in a human specimen.

As such, it cannot be predicted from Maatman et al. that kidney disease can be diagnosed by detecting L-FABP in a human specimen. Therefore, contrary to the Examiner's remarks, Maatman et al. does not provide any motivation whatsoever for the skilled artisan to use L-FABP to diagnose or prognose kidney disease in the method of Gorski et al.

IV(B). SIMON ET AL. FAILS TO REMEDY THE LACK OF MOTIVATION

The fact that Maatman et al. merely speculates as to the function of L-FAPB may prevent nephrotoxicity by binding to drugs and causing their excretion from the liver, and that this

speculation does not in any way suggest that there is a correlation between the levels of L-FABP and the diagnosis of kidney diseases was pointed out to the Examiner in the Reply filed on July 22, 2003.

In order to make up for the deficiencies of Maatman et al., the Examiner adds the new reference of Simon et al. to the combination of Gorski et al. and Maatman et al. In particular, the Examiner relies on Simon et al. to teach "experimental analysis of liver fatty acid binding protein and its function to suppress expression in the proximal nephron (kidney)." See, page 9 of the Office Action. Further, on page 4 of the Office Action, the Examiner states that Simon et al. demonstrates that L-FABP mediates suppression in the stomach, liver, and kidney, and "represents a target for identifying transcription factors that regulate gene expression."

Simon et al. utilizes L-FABP as a model for investigating the molecular mechanisms that regulate gene transcription within and between epithelial cells that line the intestine and the nephrons of the kidney. Simon et al. discloses that a DNA sequence existing in the upstream region of the L-FABP gene (which includes a sequence designated as "heptad repeat") has a function of inhibiting expression of L-FABP in mouse kidney (more specifically, tubular epithelial cells of the proximal nephron) and other organs by binding nucleic protein. Simon et al. suggests that the "heptad repeat" represents a target for identifying transcription factors

that regulate gene expression. See, page 10662, left column, lines 4-3 from the bottom of Simon et al.

However, Simon et al. merely provides a basic finding concerning control of transcription of L-FABP gene. Applicants note that, in a general sense, it is known in the art of biochemistry that gene expression is modulated up and down by transcription factors. In this regard, it is well known in the art that many genes contain binding sites for transcription factors, but the discovery of these sites, without more, does not reveal the function of these genes. The Examiner's rationale appears to be that it would have been "obvious to try" L-FABP as a marker for kidney disease since the expression of L-FABP is modulated in the kidney. Such a rationale is clearly improper:

> The admonition that "obvious to try" is not the standard under § 103 has been directed mainly at two kinds of error…. [W]hat was "obvious to try" was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it.

In re O'Farrell, 853 F.2d 894, 903 (Fed. Cir. 1988). In the instant case, Simon's discovery of a heptad repeat in the nucleotide sequence of L-FABP merely presents a field of experimentation for the role of L-FABP in the kidney. Simon's

discovery does not, on the other hand, disclose or suggest a role for L-FABP in kidney diseases. Simon et al. does not even provide general guidance as to a function, if any, of L-FABP in the kidney. In other words, Simon et al. does not make any connection between an increase in L-FABP protein and kidney disease.

For these reasons, the Examiner's combination of Simon et al. with Gorski et al. and Maatman et al. is improper. The skilled artisan simply has no motivation to combine these references. Further, the skilled artisan would never predict from Simon et al. the relationship between L-FABP and the diagnosis or prognosis of kidney disease, even in combination with the other cited references.

IV(C). GORSKI ET AL. TEACHES AWAY FROM THE PRESENT INVENTION

Finally, there is no motivation for the skilled artisan to combine the references, because Gorski et al. teaches away from the present invention. The Examiner is reminded that it is well settled in patent law that it is improper to combine references where the references teach away from their combination. In re Grasselli, 713 F.2d 731 (Fed. Cir. 1983). As discussed in detail below, Gorski et al. teaches away from the present invention in two distinct and powerful ways. First, Gorski et al. teaches the skilled artisan away from use of L-FABP as a marker for kidney disease. Second,

Gorski et al. teaches the skilled artisan away from replacing the H-FABP disclosed therein with the L-FABP disclosed in Maatman et al. As such, the skilled artisan would not be motivated to combine Gorski et al. with the other cited references.

IV(C)(i). Gorski et al. teaches away from use of FABP as a marker for kidney disease

In discussing Gorski et al., the Examiner writes, "Plasma FABP concentration is shown to markedly increase in patients with chronic renal failure. The findings suggest that the kidney plays a dominant role in the clearance of plasma FABP (citations omitted)." See, page 4, lines 1-3 of the Office Action. In doing so, the Examiner implies that Gorski et al. teaches that FABP may be used as a marker for kidney disease. Applicants respectfully disagree. Gorski et al. provides no suggestion whatsoever that FABP may be used as a diagnostic marker for kidney disease, and further teaches away from using a FABP as a marker for any disease other than myocardial infarction.

As discussed in detail in Appendix I, the FABP studied by Gorski et al. is H-FABP. Gorski et al. discloses that H-FABP is released from the heart after the onset of infarction. Based upon this finding, Gorski et al. suggests that H-FABP can serve as a marker for diagnosis of myocardial infarction. However, there is no

suggestion at all in Gorski et al. that FABP may serve as a marker for any other disease. In fact, throughout the description of Gorski et al., from the object of the study to the conclusion, only H-FABP is treated as a marker for myocardial infarction. Accordingly, there is no suggestion for using FABP in the diagnosis or prognosis of kidney disease in humans.

On page 193, right column, lines 7-2 from the bottom, Gorski et al. discusses the "usefulness of the plasma FABP[2] concentration as an early biochemical marker for myocardial infarction diagnosis", and then, on page 194, left column, lines 4-8, discusses the object of the study: "It is obvious that any change in the clearance rate of FABP[3] would affect its plasma concentration, and thus may lead to erroneous interpretation." The skilled artisan would understand that the "erroneous interpretation" referred to in the above sentence means an erroneous interpretation in the diagnosis of myocardial infarction. On page 194, left column, lines 18-30, Gorski et al. further discloses:

> Unfortunately, preinfarcation data on plasma FABP[4] in this patient were not available. Low-molecular-mass proteins such as FABP[5] and myoglobin are cleared mostly by the kidney. As it remains an open question whether, and, if so, to what extent and insufficiency of the kidneys

[2] The FABP referred to here by Gorski et al. is H-FABP.
[3] The FABP referred to here by Gorski et al. is H-FABP.
[4] The FABP referred to here by Gorski et al. is H-FABP.

affects the plasma FABP[6] concentration in patients with heart and skeletal muscles intact, we studied plasma FABP[7] and myoglobin in patients with chronic renal failure.

As is clear from the above disclosures, Gorski's object in measuring H-FABP in the plasma was to obtain an answer to the question of "whether, and, if so to what extent, an insufficiency of the kidneys" affects the plasma H-FABP concentration in order to **prevent an erroneous interpretation in the diagnosis of myocardial infarction in patients with renal insufficiency** (e.g., patients with chronic renal failure). Furthermore, in discussing the data shown in Table 1 (page 194), Gorski et al. states, "The present data are first to show that plasma FABP[8] concentration is markedly increased in patients with chronic renal failure and normal heart function, similar to that found for myoglobin...," and "[t]hese findings suggest that the kidneys play a more dominant role in the clearance of plasma FABP[9] than of myoglobin." See, page 194, right column, lines 15-20 and page 194, right column, lines 35-38, respectively.

Thus, contrary to the Examiner's statements, Gorski's findings do not suggest that the kidney plays a dominant role in the clearance of L-FABP. Instead, Gorski et al. merely states that that the value of plasma H-FABP is increased in patients with renal

---

[5] The FABP referred to here by Gorski et al. is H-FABP.
[6] The FABP referred to here by Gorski et al. is H-FABP.
[7] The FABP referred to here by Gorski et al. is H-FABP.

failure, and that this value is more affected by renal insufficiency than that of myoglobin. Gorski et al. concludes that clearance of H-FABP by the kidneys could produce a false diagnosis of myocardial infarction. As such, Gorski et al. teaches the skilled artisan away from using FABP as a marker for any kidney disease, since it is unknown how the kidneys would affect the concentration of other FABPs (i.e., non-H-FABP types) in the plasma. Accordingly, the Examiner's implication that Gorski et al. teaches that FABP may be used as a marker for kidney disease is clearly erroneous.

IV(C)(ii). Gorski et al. teaches the skilled artisan away from replacing the H-FABP disclosed therein with the L-FABP disclosed in Maatman et al.

Further, the skilled artisan would not be motivated to replace the H-FABP disclosed in Gorski et al. with the L-FABP used in the present invention, because Gorski et al. explicitly teaches that there is no correlation between the concentration of FABP and other well known kidney disease markers, such as urea and creatinine.

Specifically, on page 194, right column, lines 10-14, Gorski et al. states, "**Neither plasma FABP[10] nor plasma myoglobin concentrations showed a correlation with the period of dialysis or**

[8] The FABP referred to here by Gorski et al. is H-FABP.
[9] The FABP referred to here by Gorski et al. is H-FABP.
[10] The FABP referred to here by Gorski et al. is H-FABP.

urea or creatinine concentration in plasma (emphasis added)." It is well known in the art that creatinine and urea are widely used as markers for testing kidney sufficiency. Any person skilled in the art could easily extrapolate from the findings of Gorski et al. that there is no correlation between H-FABP levels in plasma and kidney disease, let alone L-FABP levels and kidney disease. Thus, the skilled artisan is taught away from using FABP as a marker for kidney disease.

It should also be noted that Gorski et al. concludes:

> Serial monitoring of the plasma FABP[11] concentration can also be used to estimate infarct size. However, our results indicate that if the myocardial infarction occurred in a patient with chronic renal failure, the plasma FABP[12] concentration would be relatively higher than in a patient with intact kidneys, thus leading to overestimation of infarct size…In conclusion, our data indicates that in patients with chronic renal failure the plasma concentrations of the biochemical markers FABP[13] and myoglobin each are markedly increased. Thus, caution must be taken when using these marker proteins for early diagnosis of myocardial infarction, in case of renal insufficiency, as the preinfart plasma concentration is very likely to be already high.

See, page 195, left column, lines 12-35.

---

[11] The FABP referred to here by Gorski et al. is H-FABP.
[12] The FABP referred to here by Gorski et al. is H-FABP.
[13] The FABP referred to here by Gorski et al. is H-FABP.

As is clear from this passage, Gorski's conclusions only speak to the correlation between H-FABP and the diagnosis of myocardial infarction, and the use of H-FABP as a marker for myocardial infarction. In other words, there is simply no suggestion to use FABP (either H-FABP or L-FABP) as a marker for the diagnosis or prognosis of kidney disease in humans. Further, given the lack of correlation between creatinine and urea levels with FABP, the skilled artisan would not be motivated to change the subject matter of Gorski's study from "diagnosis of myocardial infarction" to "diagnosis of kidney disease" either. Thus, Gorski et al. merely discloses or suggests the diagnosis of myocardial infarction using H-FABP as a marker, but never teaches or suggests using any FABP in the diagnosis of any other disease, such as kidney disease.

IV(D). SUMMARY

In summary, there is no motivation for the skilled artisan to combine the primary references relied upon by the Examiner (i.e., Gorski et al., Maatman et al., and Simon et al.). None of the references suggest a role for FABP (whether L-FABP or H-FABP) in the diagnosis or prognosis of kidney disease. Thus, even if the references were combined, for example by replacing the H-FABP of Gorski et al. with the L-FABP of the Maatman et al. or Simon et

al., the skilled artisan would not end up with the method of the present invention. Specifically, since none of the references suggest a correlation between kidney disease and L-FABP, and given the fact that Gorski et al. teaches away from use of FABP as a marker for diseases other than myocardial infarction, the skilled artisan would never reach the idea of the present invention.

(V) NO REASONABLE EXPECTATION OF SUCCESS DUE TO DIFFICULTY IN REPLACING H-FABP WITH L-FABP

On page 4, lines 4-7 of the Office Action, the Examiner writes, "Gorski et al. differ from the instant invention in not specifically teaching the detection of liver-type fatty acid binding protein. However, Maatman et al. identified the liver-type fatty acid binding protein utilized in the instant invention." The Examiner proceeds to replace the H-FABP disclosed in Gorski et al. with the L-FABP disclosed in Maatman et al.

The Examiner's "picking and choosing" of L-FABP to replace H-FABP of Gorski et al. is clearly improper. The Examiner is reminded that it is well settled in patent law that there must be some motivation to combine the references, either from the nature of the problem to be solved, the teachings of the prior art, and/or the knowledge of persons of ordinary skill in the art. In re Roufett, 149 F.3d 1350 (Fed. Cir. 1998). Further, the fact that references

could be combined or modified is not proper motivation. Instead, the prior art must suggest the desirability of the combination. In re Mills, 916 F.2d 680 (Fed. Cir. 1990).

Given the disclosure of Gorski et al., no person skilled in the art would have ever replaced H-FABP in Gorski et al. with L-FABP. As discussed above, Gorski et al. states on page 194, right column, lines 35-38, "These findings suggest that the kidneys play a more dominant role in the clearance of plasma FABP[14] than of myoglobin." The skilled artisan would understand from this statement that, even with proteins having a similar molecular weight such as H-FABP and myoglobin, clearance thereof in the kidney is not necessarily the same or similar. Accordingly, the skilled artisan would not be motivated to replace the H-FABP of Gorski et al. with another FABP.

Further, it would be difficult, if not impossible, to predict any success in the use of the L-FABP in the diagnosis of kidney disease in humans. In this regard, Applicants respectfully point out that it has been well known in the art for long time that the kidney participates in the clearance of not only H-FABP but also other low molecular weight proteins. This fact is also mentioned in Gorski et al., who states, "[L]ow-molecular-mass proteins such as FABP and myoglobin are cleared mostly by the kidney." See, page

194, left column, lines 20-23. Nevertheless, a satisfactory test method suitable for the diagnosis or prognosis of kidney disease has never been found.

Under these circumstances, the present inventors have intensively studied and have newly found that the occurrence of the L-FABP derived from the proximal tubule of the kidney is closely correlated with kidney disease. By focusing on L-FABP, a new method for the diagnosis or prognosis of kidney diseases was established. The method for the diagnosis of kidney disease according to the present invention is very important and very valuable in the determination of the most suitable therapeutic method for the treatment of kidney disease, such as steroid therapy, which is sometimes associated with undesirable side effects. Furthermore, the present invention can effectively be applied to the prognosis of kidney disease, which has hitherto been difficult by known methods. Such excellent effects of the present invention have never been taught or even suggested by the cited references.

(VI) UNEXPECTED SUPERIOR EFFECTS OF THE PRESENT INVENTION

As discussed above, it has been well known in the art for a long time that the kidney participates in clearance of low

[14] The FABP referred to here by Gorski et al. is H-FABP.

molecular proteins. However, it does not follow that those low molecular proteins can be used as a marker for diagnosis of kidney disease. In fact, only a few materials have been shown to be useful as a marker for diagnosis of kidney disease. These are, for example, creatinine, urea nitrogen, total urinary protein, NAG (N-acetyl-ß-D-glucosamidase), etc. Accordingly, there has been a long-standing need in the art to develop a new method for diagnosis of kidney disease. Further, since the prognosis of kidney disease is very important for determining the most suitable method for treatment, a new method for the prognosis has earnestly been desired in the art. Nevertheless, no satisfactory method for the diagnosis or prognosis of kidney disease has ever been disclosed prior to the filing of the present patent application. In fact, the present invention can be used for the prognosis of kidney disease, which hitherto has been very difficult. Hence, the present invention is extremely effective and useful in a practical standpoint.

The effectiveness and usefulness of the present invention are clear from the data published in Kamijo et al. (*J. Lab. Clin.* Med. 143(1); 23-30, 2004). The Examiner will recall that Kamijo et al. was discussed during the interview; a copy thereof is attached hereto as **Exhibit 1**.

The clinical test results of the present invention in patients with chronic renal disease are shown in Table III and page 26, left column, last line to the right column, line 8 of Kamijo et al., wherein the correlation of the method of the present invention with the progression rate of renal disease was statistically analyzed. The "F ratio" in Table III means correlation with "progression rate." This means that the larger the F ratio, the stronger the correlation. As is clear from the test results, among various diagnostic markers, only L-FABP had a statistically significant high F ratio, which proves that L-FABP has a very strong correlation with the progression rate of renal disease.

In the abstract, Kamijo et al. states, "The results showed that urinary L-FABP reflected the clinical prognosis of chronic renal disease. Urinary L-FABP may be a clinical marker that can help predict the progression of chronic glomerular disease." See, page 23, abstract, lines 19-22. Thus, it has been confirmed that the method of the present invention is superior and can also be applied to prognosis of kidney disease.

(VIII) REJECTIONS OF CLAIMS 9, 14, 15, 19, AND 21

Kimura et al. discloses merely that male rat kidney contains H-FABP and a protein obtained by modifying α2u-globulin. Galaske et al. is concerned with a model for anti-GBM nephritis and

discloses a method for preparing the model for nephritis. Finally, Zuk et al. is concerned with an immunoassay for detecting organic materials and discloses reagents or kits for the assay.

As discussed above, the present invention is not obvious over the primary combination of Gorski et al., Maatman et al. and Simon et al. The addition of Kimura et al., Galaske et al., and/or Zuk et al. fails to remedy the deficiencies of the primary references. Specifically, the additional references do not teach or suggest the use of L-FABP for diagnosis or prognosis of kidney disease, even when combined with the primary references. Thus, the present invention is not obvious over the combination references.

(IX) RESPONSE TO ARGUMENTS

On page 9 of the Office Action, the Examiner states, "In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references." Applicants agree that, when an obviousness rejection is based upon a combination of references, nonobviousness is properly shown only over the combination of references. In re Keller, 642 F.2d 413 (CCPA 1981). The Examiner's reliance on In re Keller in the instant application is misplaced however.

In In re Keller, the United States Court of Customs and Patent Appeals upheld the rejection of the claims of reissue application serial No. 865,610 for being obviousness over a combination of Keller and Walsh and/or Berkovits and Walsh. To rebut the *prima facie* case of obviousness established by the examiner, appellant filed an affidavit by an expert in the relevant field of art attacking *only* the secondary Walsh reference. Appellant offered no other rebuttal evidence. The court reasoned that Appellant failed to rebut the *prima facie* case of obviousness because the expert affidavit was only concerned with whether the Walsh reference suggested the reissue invention, rather than whether Keller in view of Walsh or Berkovits in view of Walsh references suggested the invention of the reissue application. In doing so, the court pronounced, "[O]ne cannot show non-obviousness by attacking references individually where, as here, the rejections are based on a combination of references." Id. at 426.

It must be noted, however, that the court emphasized that the examiner *had already established a prima facie case of obviousness* because the references were properly combined. Id. at 425. As such, a proper response to an examiner's obviousness rejection may include arguments that an examiner has failed to establish a *prima facie* case of obviousness because there is no suggestion or motivation to combine the references *in the first place*. "In

determining the propriety of the Patent Office case for obviousness *in the first instance*, it is necessary to ascertain whether or not the reference teachings would appear to be sufficient for one of ordinary skill in the relevant art having the reference before him to make the proposed substitution, combination, or other modification (emphasis added)." In re Linter, 458 F.2d, 1013, 1016 (CCPA 1972). In such a situation, it is proper to discuss the disclosure of each reference individually in order to argue that the skilled artisan would not combine the references in the first place. "Facts established by rebuttal evidence must be evaluated along with the facts on which the conclusion of obviousness was reached, not against the conclusion itself." U.S. Pat. & Trademark Off., Manual Pat. Examining Proc. § 2142 (8th ed. Rev. 2 2004).

In summary, therefore, Applicants respectfully disagree that the arguments submitted in the Reply under 37 C.F.R. § 1.111 filed on July 22, 2003 are improper. Specifically, Applicants disagree that the skilled artisan would be motivated to combine the Maatman et al. and Gorski et al. references. For all of the reasons discussed below, it is apparent that the skilled artisan would not be motivated to combine the references cited by the Examiner, and further, that even a combination thereof would still fail to disclose or suggest the present invention.

Summary

Applicants respectfully submit that the above remarks fully address and overcome the outstanding rejections. For the foregoing reasons, Applicants respectfully request the Examiner to withdraw all of the outstanding rejections, and to issue a Notice of Allowance indicating the patentability of the present claims. Early and favorable action of the merits of the present application is thereby respectfully requested.

Pursuant to the provisions of 37 C.F.R. §§ 1.17 and 1.136(a), the Applicants hereby petition for an extension of two (2) months to October 14, 2004, in which to file a reply to the Office Action. The required fee of $430.00 is enclosed herewith.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
Gerald M. Murphy, Jr., #28,977
P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

KLR

GMM/KLR
0020-4710P
Attachments:
Appendix I
Kamijo et al., *J. Lab. Clin. Med.*, 143:23-30, 2004
(Exhibit 1)

APPENDIX 1

As discussed in detail in the Reply under 37 C.F.R. § 1.111 filed on July 22, 2003 and during the interview held on August 17, 2004, the fatty acid-binding protein (FABP) of Gorski et al. is H-FABP (heart-type FABP), which is clearly distinguishable from L-FABP (liver-type FABP) of the present invention. During the interview, the Examiner requested that the differences between H-FABP and L-FABP be clearly presented in the record. The following discussion therefore is in response to the Examiner's request.

(1) FABP in Gorski et al. is heart-type FABP

From the very opening phrase of Gorski et al., it is clear that the FABP disclosed therein is H-FABP:

> The soluble cytoplasm of most cells contains low-molecular-mass (14-15 kDa) proteins able to bind long-chain unesterified fatty acids. Of these so-called fatty acid-binding proteins (FABP), nine different types have been identified [1, 2]. Heart and skeletal muscles contain the same type of FABP (referred to as heart-type (H)-FABP [1, 2], but its concentration in the heart is several fold higher than that in the skeletal muscles [3]. The concentration of FABP in the plasma of healthy persons is relatively low (2-6 $\mu \cdot L^{-1}$)[4]. FABP is released from the heart early after the onset of infarction, whereafter its plasma concentration increases many fold [3-6]. Increased excretion of FABP in urine

> also occurs after infarction [5,7]. Several recent studies indicate the usefulness of the **plasma FABP concentration as an early biochemical marker for myocardial infarction diagnosis** [3,5,7].

See, page 193, right column (emphasis added).

As is clear from this opening passage, the FABP focused on by Gorski et al. is an FABP which functions as a marker for myocardial infarction diagnosis and is released from the heart after the onset of infarction. This FABP is an FABP existing in the heart, that is, H-FABP. Thus, the skilled artisan can readily understand that Gorski et al. is directed to the study of H-FABP as a marker for myocardial infarction.

Further, it is clear that the FABP studied by Gorski et al. is H-FABP in view of the method used by Gorski et al. to measure FABP. Specifically, on page 194, middle column, lines 13-15, Gorski et al. describes the experimental method as, "Plasma FABP concentration as measured by a sensitive **noncompetitive sandwich ELISA** [4] (emphasis added)." The reference [4] cited by Gorski et al. is Wodzig et al, *Ann. Clin. Biochem.*, 34:263-268, 1997.[15]

[15] Wodzig et al. was submitted as Exhibit 1 attached to the Reply filed on July 22, 2003, and has already been disclosed in an Information Disclosure Statement filed on October 3, 2002. Thus, another copy of Wodzig et al. is not attached hereto. If the Examiner needs another copy of Wodzig et al., however, she is respectfully requested to contact the undersigned.

In Wodzig et al., the method for measuring plasma FABP is disclosed. Specifically, in the summary, pages 1-3, Wodzig et al. discloses:

> To allow a more rapid determination of heart-type fatty acid-binding protein (FABP) concentration in plasma a **direct non-competitive (sandwich-type) ELISA was developed which uses high-affinity monoclonal antibodies to FABP** (emphasis added).

Furthermore, on page 264, left column, lines 27-43, Wodzig et al. adds:

> **For measurement of FABP in serum or plasma, a direct non-competitive ELISA of the antigen capture type (sandwich ELISA) was developed, based on the use of monoclonal antibodies (mAb).** Thirteen **mAbs**, all of subtype IgG1 and **directed against purified human heart-type FABP** were raised by the classical hybridoma technology and characterized by surface plasma resonance analysis using a Pharmacia BIA core biosensor, as described in detail elsewhere. The **mAbs** recognized five distinct (three independent and two overlapping) epitopes on human FABP and **showed no cross-reactivity with human intestinal-type and human live-type FABP.** Seven of the mAbs were selected...(emphasis added).

As noted in the above passage, the monoclonal antibodies (mAbs) utilized in the non-competitive sandwich ELISA are directed against human H-FABP. These monoclonal antibodies show no cross-reactivity with human L-FABP. Thus, the direct non-competitive

(sandwich-type) ELISA disclosed in Wodzig et al. and utilized by Gorski et al. measures only H-FABP concentration in plasma. In other words, since the monoclonal antibodies do not cross-react with L-FABP, it is not possible to measure L-FABP with this method. In view of Wodzig et al., therefore, the "FABP" referred to by Gorski et al. is clearly H-FABP.

(2) Differences between H-FABP and L-FABP

H-FABP and L-FABP are clearly different proteins and are recognized by the skilled artisan as such, for reasons summarized in the following table.

	L-FABP (LIVER-TYPE FABP)	**H-FABP (HEART-TYPE FABP)**
Proteins and genes	L-FABP, H-FABP, and other FABPs are each different proteins, having an amino acid sequence homology of only about 38 to 70%; It is also well known that they have different nucleotide sequences.	
Tissue occurrence	liver, intestine, kidney, etc.	heart, skeletal muscle, kidney, etc.
Expression in the kidney (cf. Example 3 and Fig. 1 of the instant specification)	In human kidney tissue, L-FABP exists at the proximal tubule.	In human kidney tissue, H-FABP exists mainly at the distal tubule.

These differences in H-FABP and L-FABP are supported by Van Nieuwenhovern et al., *Lipids*, Vol. 31 Suppl: S223-S227, 1996 (cited as [2] in Gorski et al.) and Veerkamp et al., *Prog. Lipid Res.*,

34(1):17-52, 1995.[16] Van Nieuwenhovern et al. lists the various FABPs on page s225, Table 2. Veerkamp et al. lists the various FABPs on page 21, Table 3. In both of these tables, H-FAPB and L-FABP are classified as different proteins. Furthermore, Veerkamp et al. discloses the amino acid sequence alignment between human heart-type FABP and human liver-type FABP on page 23, Figure 2. It is clear from the alignment that the two FABP proteins do not share a high homology.

(3) Summary

In summary, it shall be understood by the skilled artisan that the FABP measured by noncompetitive sandwich ELISA and discussed in the experimental results of Gorski et al. is H-FABP, even though Gorski et al. merely refers to the H-FABP as simply "FABP." Accordingly, it is clear that the H-FABP studied by Gorski et al. is clearly different from the L-FABP to be measured by the present invention.

[16] Both of these journal articles have already been made of record in the present application in an Information Disclosure Statement (IDS) and attached as Exhibits 2 and 3, respectively, to the Reply filed on July 22, 2003. Thus, copies of these articles are not attached hereto. If the Examiner needs copies of these articles, however, she is respectfully requested to contact the undersigned.

Urinary fatty acid–binding protein as a new clinical marker of the progression of chronic renal disease

ATSUKO KAMIJO, KENJIRO KIMURA, TAKESHI SUGAYA, MASAYA YAMANOUCHI, AKIHISA HIKAWA, NORIHITO HIRANO, YASUNOBU HIRATA, ATSUO GOTO, and MASAO OMATA

TOKYO, KAWASAKI, OSAKA, and TOCHIGI, JAPAN

Previous studies have indicated that in massive proteinuria, free fatty acids (FFAs) bound to albumin were overloaded in the proximal tubule and exacerbated tubulointerstitial damage. Liver-type fatty acid–binding protein (L-FABP) is an intracellular carrier protein of FFAs that is expressed in the proximal tubule of human kidney. We sought to evaluate urinary L-FABP as a clinical marker in chronic renal disease. Urinary L-FABP was measured in patients with nondiabetic chronic renal disease (n = 120) with the use of a newly established ELISA method. We then monitored these patients for 15 to 51 months. Clinical data were analyzed with multivariate analysis. Urinary L-FABP was correlated with urinary protein, urinary α_1-microglobulin, and serum creatinine concentrations. Urinary L-FABP at the start of follow-up (F = 17.1, r = .36, P < .0001) was selected as a significant clinical factor correlated with the progression rate, defined as a slope of a reciprocal of serum creatinine over time. We next selected the patients with mild renal dysfunction (n = 35) from all 120 patients and divided them into 2 groups according to progression rate: the progression group (n = 22) and the nonprogression group (n = 13). Serum creatinine and urinary protein concentrations and blood pressure at the start of follow-up were higher in the progression group than in the nonprogression group, although we detected no significant difference between the 2 groups. Urinary L-FABP was significantly higher in the former group than in the latter (P < .05). The results showed that urinary L-FABP reflected the clinical prognosis of chronic renal disease. Urinary L-FABP may be a clinical marker that can help predict the progression of chronic glomerular disease. (J Lab Clin Med 2004;143:23-30)

Abbreviations: BSA = bovine serum albumin; ELISA = enzyme-linked immunosorbent assay; FFA = free fatty acid; L-FABP = liver-type fatty acid–binding protein; mAb = monoclonal antibody; α_1-MG = α_1-microglobulin; NAG = *N*-acetyl-β-D-glucosaminidase; PBS = phosphate-buffered saline solution

Increasing clinical and experimental evidence shows that the progressive nature of chronic glomerular disease depends significantly on tubulointerstitial involvement.[1] Recent studies have shown that urinary protein has renal toxicity and contributes to the progression of renal damage by causing tubulointerstitial disease.[2-6] FFAs bound to albumin[7,8] may play a role in the generation of tubulointerstitial disease. In massive proteinuria, FFAs are overloaded in the proximal tubule and induce inflammatory factors such as macrophage chemotactic factors,[9] which in turn aggravate urinary protein–related tubulointerstitial damage.[10-12] We showed that FFAs may be responsible for 1 mechanism leading to tubulointerstitial damage seen in massive proteinuria.[13] FFAs are overloaded in the proximal tubule not only in massive proteinuria but also in various other kinds of stresses to the proximal tubule such as ischemia[14] and toxic insults,[15] both of which have been implicated in the progression of renal disease.

FFAs loaded in the proximal tubule are believed to

From the Second Department of Internal Medicine, University of Tokyo; Internal Medicine, St Marianna University School of Medicine; CMIC Co, Ltd; Tanabe Seiyaku Co Ltd; and Eiken Chemical Co Ltd.

Submitted for publication August 12, 2003; revision submitted August 12, 2003; accepted for publication August 25, 2003.

Reprint requests: Kenjiro Kimura, MD, PhD, Professor of Medicine, Nephrology, and Hypertension, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-Ku, Kawasaki 216-8511, Japan; e-mail: kimura@marianna-u.ac.jp.

0022-2143/$ – see front matter

doi:10.1016/j.lab.2003.08.001

Table I. Clinical and laboratory findings

Variable	n = 120
Age (yr)*	45 ± 15
Sex (%)	
Male	77 (64)
Female	43 (36)
Mean blood pressure (mm Hg)*	96 ± 14
Serum creatinine (mg/dL)*	1.0 ± 0.4
Total cholesterol (mg/dL)*	202 ± 41
Triglyceride (mg/dL)*	143 ± 93
Urinary protein (g/g · cr)*	1.1 ± 1.3
Urinary NAG (IU/g · cr)*	7.6 ± 6.1
Urinary α1-MG (mg/g · cr)*	9.7 ± 9.9
Urinary L-FABP (μg/g · cr)*	30.1 ± 38.9
Renal biopsy (%)	89 (74)
Minor glomerular abnormalities	15
Mesangial proliferative glomerulonephritis	49
Membranous nephropathy	6
Focal segmental glomerulosclerosis	18
Membranoproliferative glomerulonephritis	1
Medication†	
On	73
Off	47

*Data expressed as mean ± SD.
†Medication included an angiotensin-converting enzyme inhibitor, an angiotensin-1 receptor antagonist, and steroids.

be bound to cytoplasmic fatty acid–binding protein and transported to mitochondria or peroxisomes, where they are metabolized by β-oxidation. In the human proximal tubules, L-FABP of 14.4 kD is expressed.[16] Because renal L-FABP has not yet been investigated in patients with renal disease, we sought to clarify the clinical relevance of urinary excretion of L-FABP in chronic glomerular disease after developing specific mAbs against human L-FABP.

METHODS

Patient selection. We selected 120 nondiabetic adult patients from the outpatient clinic of the University of Tokyo Hospital between 1997 and 2000 on the basis of a serum creatinine concentration less than 2.5mg/dL. Relevant clinical parameters were monitored every month or second month for more than 15 months, with no change in medication. In each patient the diagnosis of chronic glomerular disease had been made on the basis of renal biopsy (n = 89) or clinical history, and none had clinical or laboratory evidence of underlying systemic disease. Table I summarizes the clinical and laboratory findings of the subjects. This research was carried out in accordance with the principles of the Declaration of Helsinki; informed consent was obtained and our institutional review board approved the study.

Development of specific mAbs against human L-FABP. BALB/C mice were subcutaneously injected once with 50 μg of purified recombinant human L-FABP in Freund's complete adjuvant, and the same dose was injected after 2 weeks. We prepared recombinant human L-FABP using the fusion plasmid system (pMAL-cRI).[17] Spleen cells from immunized mice were fused with the murine myeloma line P3/X63-AG8 6.5.3.[18] We selected hybridomas in a hypoxanthine aminopterin thymidine medium and screened for antibody production using an ELISA and the purified recombinant L-FABP–coated plate. We obtained 18 positive clones by limiting dilution. Expanded cultures from 2 hybridomas, FABP-2 and FABP-L, were injected into the peritoneal cavities of pristane-primed mice, after which we collected ascitic fluid and fractionated it into IgG by means of protein A column chromatography. mAb FABP-2 was conjugated to horseradish peroxidase with the use of succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate, in accordance with the instructions of the manufacturer (Pierce Chemical Co, Rockford, IL).

Establishment of ELISA for urinary L-FABP. We coated 96-well microtiter plates with 10 mg/L mAb FABP-L and incubated them overnight. Unreacted sites were blocked with PBS containing 10 g/L BSA overnight. The plates were then washed 3 times with PBS containing 0.5 g/L Tween-20 with 1 g/L BSA and dried.

We incubated 100 μL of properly diluted standards or samples in the wells of each plate at room temperature for 1 hour. They were then washed 4 times with PBS containing 0.5 g/L Tween-20 and allowed to react with 100 μL of horseradish peroxidase–conjugated FABP-2 for 1 hour. After 4 more washes, 100 μL of enzyme substrate (*O*-phenylenediamine/H_2O_2) solution was reacted at room temperature for 30 minutes, after which the reaction was terminated with the addition of 100 μL of 2 mol/L sulfuric acid. Absorbance was measured at 492 nm on a microplate reader.

We prepared standards for the assay by measuring the protein concentration of purified recombinant L-FABP using Lowry's method and adjusting to make up a series ranging from 0 to 400 ng/mL with PBS containing 10 g/L BSA.

The reference value of urinary excretion of L-FABP was set after analysis of urine samples from healthy volunteers at Eiken Chemical Co Ltd (n = 97). None of the volunteers had a history of renal disease or abnormal finding on urinalysis.

Clinical parameters of serum and ambulatory spot urine samples. Creatinine, total cholesterol, and triglyceride concentrations were measured in serum; L-FABP, creatinine, total protein, NAG, and α_1-MG concentrations were measured in urine. We used enzymatic methods to measure serum and urinary creatinine, serum cholesterol, and triglyceride; the pyrogallol red–molybdate complex method to measure urinary protein; chlorophenol red–NAG as substrate to measure urinary NAG; and the latex aggregation assay to measure α_1-MG. Serum creatinine was measured every month or second month in the study patients.

We divided the patients into 3 groups on the basis of urinary protein level: The "mild proteinuria" group (n = 76) consisted of patients with urinary protein of less than under 1 g/g · cr, the "moderate proteinuria" group (n = 33) comprised patients with a value of 1 to 3 g/g · cr, and the "heavy proteinuria" group (n = 11) consisted of patients with values of more than 3 g/g · cr. We compared 3 groups with the level of urinary L-FABP.

Clinical course and urinary L-FABP. In an effort to evaluate the progression of renal disease, we analyzed the relationship between time (months) and the reciprocal of serum creatinine.[19-26] The progression rate of renal disease was defined as a slope of the regression line. In the patients whose serum creatinine fluctuated within the normal range during the follow-up period, the progression rate was evaluated as zero because the slope of the regression line did not represent the progression of renal disease in those patients.

Moreover, we selected the patients with mild renal dysfunction whose serum creatinine values were greater than the normal range (1.2 mg/dL in men, 0.9 mg/dL in women) from all the patients shown in Table I ($n = 35$). We divided these patients into 2 groups: The first group consisted of patients showing a slope of the regression line of less than $-.001$ (dL $\cdot$ mg^{-1} $\cdot$ month^{-1}) ($n = 22$), the average of all 120 patients, and the second group comprised patients showing a slope of the regression line of more than $-.001$ (dL $\cdot$ mg^{-1} $\cdot$ month^{-1}) ($n = 13$). We compared the 2 groups with regard to clinical parameters at the start of follow-up, histologic diagnosis, presence or absence of medication, and duration of follow-up.

Statistical analysis. Data are expressed as the mean ± SD. To clarify the relationship between urinary L-FABP and clinical parameters and the relationship between the progression rate and clinical parameters, we applied multiple-regression analysis based on the stepwise method. Differences in parameters among the 3 groups were analyzed with Scheffé's multiple-comparison procedure after the Kruskal-Wallis test was performed. To compare the parameters from the 2 groups, we used the Mann-Whitney U test for unpaired data and the Wilcoxon rank-sum test for paired data. We conducted comparisons of categorical variables between the 2 groups using Fisher's exact probability test. These statistical analyses were performed with a software program for the Macintosh operating system (Stat View 5.0; SAS Institute, Inc., Cary, NC). P values of less than .05 were considered statistically significant.

RESULTS

ELISA for urinary L-FABP. The within-assay variance was between 6.1%, 4.9%, and 3.8% (coefficient of variation) when 8 replicates were performed on urine samples with L-FABP concentrations of 13.5, 45, and 125 ng/mL, respectively. We evaluated between-assay variability by measuring the same 3 samples in each plate for 10 days. The coefficients of variation were 6.6%, 4.2%, and 5.1%, respectively. The recovery of L-FABP values was between 90% and 100% when purified recombinant L-FABP was added in concentrations of 50, 100, and 200 ng/mL to urine samples. Serial dilutions of urine samples with L-FABP concentrations of 15, 94, and 270 ng/mL were linear.

The reference value of urinary excretion of L-FABP was analyzed in 26 male and 71 female volunteers. The average age of our healthy volunteers was 33.0 ± 7.5 years, and no sex-related statistical difference was detected. The reference value of urinary L-FABP was 6.5 ± 5.4 ng/mL, or 5.2 ± 3.6 μg/g · cr in healthy volunteers ($n = 97$), whereas the level of urinary L-FABP was 31.9 ± 48.0 ng/mL, or 30.1 ± 38.9 μg/g · cr in the patients with chronic glomerular disease ($n = 120$). The urinary L-FABP concentration was significantly higher in the patients with chronic glomerular disease than in healthy volunteers ($P < .0001$).

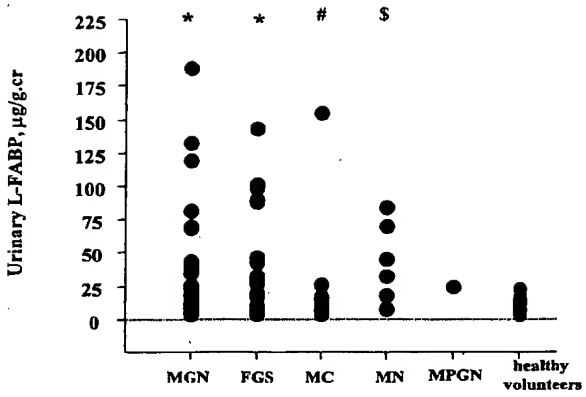

Fig 1. Distribution of urinary excretion of L-FABP in healthy volunteers and patients with various renal diseases. *MGN* = patients with mesangial proliferative glomerulonephritis; *FGS* = patients with focal segmental glomerulosclerosis; *MC* = patients with minor glomerular abnormalities; *MN* = patients with membranous nephropathy; *MPGN* = patient with membranoproliferative glomerulonephritis. *$P < .0001$ vs healthy volunteers; $$P < .0005$ vs healthy volunteers; #$P < .05$ vs healthy volunteers.

We selected the patients with chronic glomerular disease diagnosed on the basis of renal-biopsy findings ($n = 89$) (Fig 1). Compared with urinary excretion of L-FABP in healthy volunteers, urinary excretion of L-FABP was significantly increased in the patients with mesangial proliferative glomerulonephritis (25.5 ± 36.7 μg/g · cr; $P < .0001$, $n = 49$), focal segmental glomerulosclerosis (42.9 ± 42.7 μg/g · cr; $P < .0001$, $n = 18$), membranous nephropathy (43.0 ± 29.6 μg/g · cr; $P < .0005$, $n = 6$), and minor glomerular abnormalities (18.8 ± 37.4 μg/g · cr; $P < .05$, $n = 15$). The urinary L-FABP concentration in the patients with membranoproliferative glomerulonephritis was 24.6 μg/g · cr ($n = 1$).

Clinical parameters correlated with urinary L-FABP. When we conducted a stepwise regression analysis using urinary L-FABP as a dependent variable and 6 clinical independent variables (serum creatinine, serum total cholesterol, serum triglyceride, urinary protein, urinary NAG, and urinary α_1-MG), urinary L-FABP was found to be significantly correlated with urinary protein ($F = 24.2$), urinary α_1-MG ($F = 13.1$), and serum creatinine ($F = 11.9$) ($r = .76$, $P < .0001$; Table II).

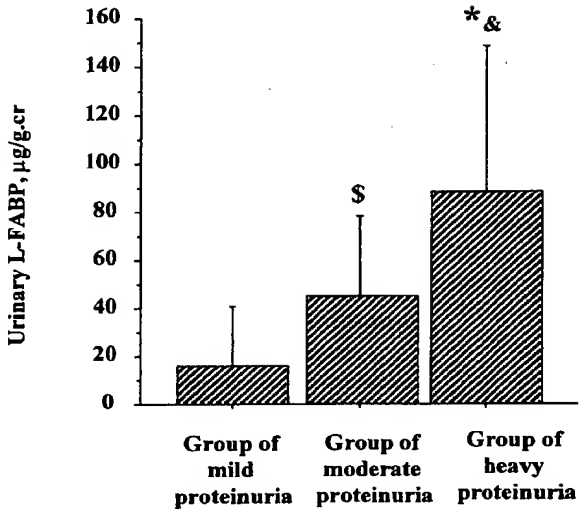

Fig 2. Urinary excretion of L-FABP in the patients divided into 3 groups on the basis of urinary protein concentration. Urinary excretion of L-FABP was significantly greater in the heavy-proteinuria group than in the mild- and moderate-proteinuria groups. Urinary excretion of L-FABP was significantly greater higher in the moderate-proteinuria group than in the mild-proteinuria group.
*$P < .0001$ vs mild-proteinuria group; $\$P < .0005$ vs the mild-proteinuria group; $\&P < .001$ vs the moderate-proteinuria group.

Table II. Stepwise regression analysis for urinary excretion of L-FABP

Independent variables	F ratio
Serum creatinine	11.9*
Total cholesterol	0.01
Triglyceride	0.23
Urinary protein	24.2*
NAG	0.03
α_1-MG	13.1*

*Statistically significant.

In the heavy-, moderate-, and mild-proteinuria groups, urinary L-FABP levels were 88.2 ± 60.4, 43.9 ± 34.4, and 15.8 ± 25.0 μg/g · cr, respectively. Urinary L-FABP levels in the heavy-proteinuria group were significantly higher than those in the moderate-proteinuria group ($p < .001$) or those in the mild-proteinuria group ($P < .0001$). Urinary L-FABP levels in the moderate-proteinuria group were significantly higher than those in the mild-proteinuria group ($P < .0005$; Fig 2).

Clinical course and urinary L-FABP. Patients were monitored for 27.3 ± 9.4 months (range 15–51 months). The slope-of-regression line was −.001 ± .004 ($dl \cdot mg^{-1} \cdot month^{-1}$). In the stepwise regression analysis with 10 clinical independent variables (urinary excretion of L-FABP, serum creatinine, serum total cholesterol, serum triglyceride, urinary protein excretion, urinary NAG, urinary α_1-MG, age, mean blood pressure, duration of follow-up) on all of the patients shown in Table 1, the progression rate was correlated only with the urinary excretion of L-FABP ($F = 17.1$, $r = .36$, $P < .0001$; Table III). Neither sex nor the presence of medication influenced the progression rate (data not shown). We selected the patients with mild renal dysfunction described above from the patients shown in Table 1 and divided them into 2 groups, the first with progression of renal disease and the second group without progression (Table IV). We confirmed that the slope-of-regression line was significantly lower in group 1 than in group 2 ($P < .0001$; Fig 3, *A* and *B*). In the first group, serum creatinine was significantly increased, from 1.7 ± .4 to 2.5 ± 1.5 mg/dL, at the end of follow-up, 26.1 ± 8.6 months ($P < .0001$; Fig 3, *C*).

Table III. Stepwise regression analysis for progression rate of renal disease

Variables	F ratio
Serum creatinine	1.5
Total cholesterol	1.5
Triglyceride	0.2
Urinary protein	2.0
NAG	0.1
α1-MG	0.2
L-FABP	17.1*
Age	0.1
Mean blood pressure	0.4
Duration of follow-up	0.0

*Statistically significant.

Table IV. Comparison of background in group 1 (progression group) and group 2 (nonprogression group)

Variable	Group 1 ($n = 22$)	Group 2 ($n = 13$)
Age (yr)*	51 ± 17	50 ± 10
Sex		
Male	17	8
Female	5	5
Follow-up (mo)*	26.1 ± 8.6	23.8 ± 12.5
Histologic diagnosis		
Minor glomerular abnormalities	2	0
Mesangial proliferative glomerulonephritis	5	7
Membranous nephropathy	1	1
Focal segmental glomerulosclerosis	6	0
Unknown	8	5

*Data expressed as mean ± SD.

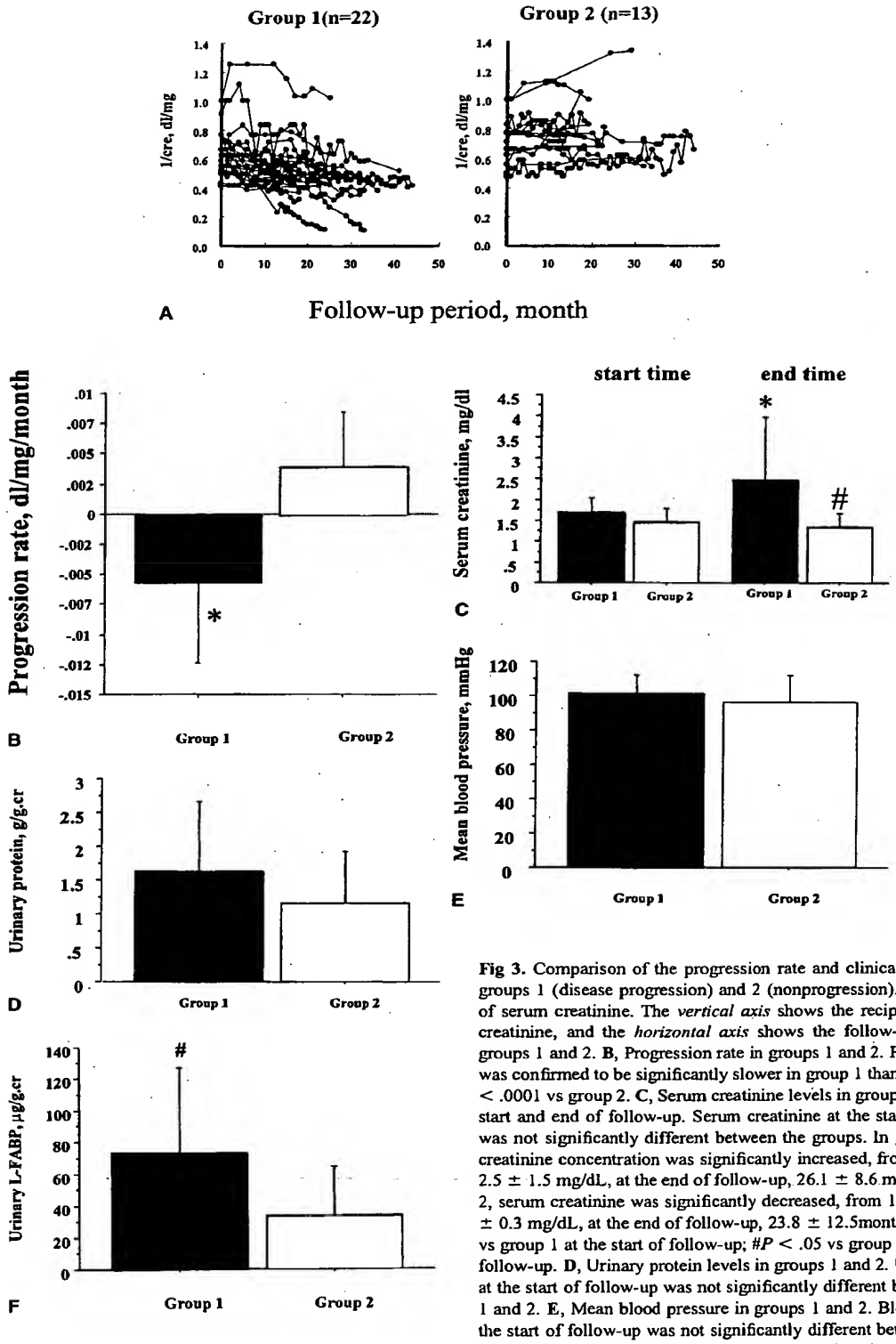

Fig 3. Comparison of the progression rate and clinical parameters in groups 1 (disease progression) and 2 (nonprogression). **A**, Reciprocal of serum creatinine. The *vertical axis* shows the reciprocal of serum creatinine, and the *horizontal axis* shows the follow-up periods for groups 1 and 2. **B**, Progression rate in groups 1 and 2. Progression rate was confirmed to be significantly slower in group 1 than in group 2. *$P < .0001$ vs group 2. **C**, Serum creatinine levels in groups 1 and 2 at the start and end of follow-up. Serum creatinine at the start of follow-up was not significantly different between the groups. In group 1, serum creatinine concentration was significantly increased, from 1.7 ± 0.4 to 2.5 ± 1.5 mg/dL, at the end of follow-up, 26.1 ± 8.6 months. In group 2, serum creatinine was significantly decreased, from 1.4 ± 0.4 to 1.3 ± 0.3 mg/dL, at the end of follow-up, 23.8 ± 12.5months. *$P < .0001$ vs group 1 at the start of follow-up; #$P < .05$ vs group 2 at the start of follow-up. **D**, Urinary protein levels in groups 1 and 2. Urinary protein at the start of follow-up was not significantly different between groups 1 and 2. **E**, Mean blood pressure in groups 1 and 2. Blood pressure at the start of follow-up was not significantly different between groups 1 and 2. **F**, Urinary L-FABP levels in groups and 2. Urinary L-FABP was significantly higher in group 1 than in group 2. #$P < .05$ vs group 2.

Meanwhile, in the second group, serum creatinine was significantly decreased, from 1.4 ± .4 to 1.3 ± .3 mg/dL, at the end of the follow-up, 23.8 ± 12.5 months ($P < .05$; Fig 3, *C*). Serum creatinine (Fig 3, *C*), urinary protein (Fig 3, *D*), and mean blood pressure (Fig 3, *E*) at the start of follow-up were higher in group 1 than in group 2, although the difference was not significant. Only urinary L-FABP was significantly higher in the former group than in the latter ($P < .05$; Fig 3, F). Sex, histologic diagnosis, the presence or absence of medication, and duration of follow-up were not significantly different between the 2 groups.

DISCUSSION

We developed specific mAbs against human L-FABP to clarify the clinical relevance of urinary L-FABP in chronic glomerular disease. We have shown urinary L-FABP to be a clinical parameter that reflects the progression of renal disease and have determined that this value was higher in the group demonstrating progression of renal disease than in the patients in whom disease was not progressing. Therefore we propose that urinary L-FABP has promise as a clinical marker to predict and monitor the progression of chronic glomerular disease. Such a predictor will contribute to the treatment of renal disease.

To investigate the clinical significance of urinary L-FABP, we measured urinary L-FABP using ambulatory spot urine samples because these samples were easy to obtain in the outpatient clinic and contamination of such samples is less than that in 24-hour urine collections. The Cockroft-Gault formula, or MDRD formula, for the evaluation of creatinine clearance is not suitable in Japanese patients, whose urinary excretion of creatinine decreases less with age than it does in Europeans and Americans.[27] We therefore defined progression of renal disease as the slope of the reciprocal of serum creatinine.[23-26]

In the human kidney, 2 types of FABP have been localized.[16] One, the liver type, is expressed in the proximal tubule; the other, a heart type, is expressed in the distal tubule. L-FABP, a small protein of 14.4 kD, is a carrier protein that transports fatty acids to mitochondria or peroxisomes, where fatty acids are metabolized by way of β-oxidation. The transcription of L-FABP gene is promoted by FFAs.[28] Recent evidence suggests that L-FABP transports FFAs from the cytosol to the nucleus[29,30] and interacts with the nuclear protein, peroxisome proliferator–activated receptor,[31] which is a nuclear target for FFAs and initiates gene expression of enzymes involved in lipid metabolism.[32,33] L-FABP may thus play a key role in fatty-acid metabolism in the proximal tubules, being induced by fatty acids per se.

As FFAs are bound to serum albumin,[7,8] filtered through glomeruli, and reabsorbed into the proximal tubule along with albumin, fatty acids are overloaded in the proximal tubule in massive proteinuria. Nonoxidized fatty acids appear to be cytotoxic by peroxidation[34,35] and induce some chemoattractants from the proximal tubules,[9] which in turn progress to tubulointerstitial damage.[13] These are supported by clinical findings that proteinuria is a major factor in the progression of renal disease.[2-6]

Tubulointerstitial inflammation induced by lipid toxicity may be provoked not only by proteinuria but also by other stresses such as ischemia[34,35] and toxins.[36,37] We therefore hypothesize that various stresses to the proximal tubule tend to overload fatty acids in the cytoplasm and thereby damage tubules with the release of inflammatory factors. In this way, tubulointerstitial inflammation is provoked and renal function deteriorates over time.

Because we detected a significant correlation of urinary L-FABP with urinary protein, we assumed that urinary protein induced excretion of L-FABP. To confirm this supposition, we prepared human L-FABP gene transgenic mice and made a protein-overload model. In control transgenic mice, human L-FABP was immunohistochemically identified in the proximal tubule and not detectable in urine. In the protein-overload model, Northern-blot analysis showed up-regulation of human L-FABP gene expression and massive amounts of L-FABP were excreted into urine (unpublished observations). The results suggest that stresses on the proximal tubules, such as urinary protein, induce up-regulation of human L-FABP gene expression and accelerate excretion of L-FABP from the proximal tubule, resulting in an increase in urinary excretion of L-FABP. From the clinical findings reported herein and the experiments involving transgenic mice, we hypothesize that a variety of stresses, such as massive proteinuria and ischemia, cause an overload of FFAs in the proximal tubules and exacerbate tubulointerstitial damage. L-FABP gene expression is increased and L-FABP accelerates fatty-acid metabolism by carrying them to mitochondria or peroxisomes. Moreover, the results suggest that L-FABP helps maintain a low level of fatty acids in the cytoplasm by being excreted from the proximal tubules into urine together with fatty acids. This mechanism should be clarified in a future study.

It is widely confirmed that quantification of urinary protein helps physicians predict the risk of disease progression and the risk of dialysis for individual patients.[5,6] However our results show that the progression rate was less correlated with urinary protein than with urinary excretion of L-FABP. Our patients' average urinary protein value was relatively modest and their

characterization might be limited. To demonstrate the exact diagnostic validity of urinary L-FABP as a predictor, we are planning a multiple-institution study.

In conclusion, urinary excretion of L-FABP may reflect various kinds of stresses that cause tubulointerstitial damage, such as those caused by urinary protein on the proximal tubule, and may therefore be unique clinical marker of the progression of chronic renal disease.

We are indebted to Drs. Masaomi Nangaku, Tomoyo Kaneko, Kazuhisa Miyashita, Naoe Suzuki, Hiroshi Satonaka, Etsu Suzuki, Akihiro Tojo, Shoko Tateishi, Kanae Kubo, Hiroko Kanda, Toshihide Mimura, Haruhiko Yoshida, and Yasushi Shiratori; Nobuko Watanabe and Shigeo Okubo for assistance with urine and serum collection; and Sanae Ogawa for assistance with laboratory technique. We also thank Yasuhiro Nomata for assistance with measurement procedures and Mitsuhiro Okada, Fumikazu Okumura, Akiko Honda, Hiromi Hase, and Shigeyoshi Oba for assistance with mouse experiments.

REFERENCES

1. Risdon RA, Sloper JC, De Wardener HE. Relationship between renal function and histological changes found in renal-biopsy specimens from patients with persistent glomerular nephritis. Lancet 1968;2:363-6.
2. Eddy AA, McCulloch L, Liu E, Adams J. A relationship between proteinuria and acute tubulointerstitial disease in rats with experimental nephrotic syndrome. Am J Pathol 1991;138:1111-23.
3. Eddy AA. Experimental insights into the tubulointerstitial disease accompanying primary glomerular lesions. J Am Soc Nephrol 1994;5:1273-87.
4. Peterson JC, Adler S, Burkart JM, Greene T, Hebert LA, Hunsicker LG, et al. Blood pressure control, proteinuria, and the progression of renal disease. The Modification of Diet in Renal Disease Study. Ann Intern Med 1995;123:754-62.
5. Remuzzi G, Ruggenenti P, Benigni A. Understanding the nature of renal disease progression. Kidney Int 1997;51:2-15.
6. Remuzzi G, Bertani T. Pathophysiology of progressive nephropathies. N Engl J Med 1998;339:1448-56.
7. Spector AA. Fatty acid binding to plasma albumin. J Lipid Res 1975;16:165-79.
8. Hamilton JA, Era S, Bhamidipati SP, Reed RG. Locations of the three primary binding sites for long-chain fatty acids on bovine serum albumin. Proc Natl Acad Sci U S A 1991;88:2051-4.
9. Kees-Folts D, Sadow JL, Schreiner GF. Tubular catabolism of albumin is associated with the release of an inflammatory lipid. Kidney Int 1994;45:1697-709.
10. Arici M, Chana R, Lewington A, Brown J, Brunskill NJ. Stimulation of proximal tubular cell apoptosis by albumin-bound fatty acids mediated by peroxisome proliferator activated receptor-gamma. J Am Soc Nephrol 2003;14:17-27.
11. Thomas ME, Schreiner GF. Contribution of proteinuria to progressive renal injury: consequences of tubular uptake of fatty acid bearing albumin. Am J Nephrol 1993;13:385-98.
12. Thomas ME, Morrison AR, Schreiner GF. Metabolic effects of fatty acid–bearing albumin on a proximal tubule cell line. Am J Physiol 1995;268:F1177-84.
13. Kamijo A, Kimura K, Sugaya T, Yamanouchi M, Hase H, Kaneko T, et al. Urinary free fatty acids bound to albumin aggravate tubulointerstitial damage. Kidney Int 2002;62:1628-37.
14. Saluja I, Song D, O'Regan MH, Phillis JW. Role of phospholipase A2 in the release of free fatty acids during ischemia-reperfusion in the rat cerebral cortex. Neurosci Lett 1997;233: 97-100.
15. Walker PD, Shah SV. Evidence suggesting a role for hydroxyl radical in gentamicin-induced acute renal failure in rats. J Clin Invest 1988;81:334-41.
16. Maatman RG, van de Westerlo EM, van Kuppevelt TH, Veerkamp JH. Molecular identification of the liver- and the heart-type fatty acid–binding proteins in human and rat kidney. Use of the reverse transcriptase polymerase chain reaction. Biochem J 1992;288:285-90.
17. Nagai K, Thogersen HC. Generation of beta-globin by sequence-specific proteolysis of a hybrid protein produced in Escherichia coli. Nature 1984;309:810-2.
18. Kearney JF, Radbruch A, Liesegang B, Rajewsky K. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J Immunol 1979;123:1548-50.
19. Muray S, Martin M, Amoedo ML, Garcia C, Jornet AR, Vera M, et al. Rapid decline in renal function reflects reversibility and predicts the outcome after angioplasty in renal artery stenosis. Am J Kidney Dis 2002;39:60-6.
20. Lin JL, Ho HH, Yu CC. Chelation therapy for patients with elevated body lead burden and progressive renal insufficiency. A randomized, controlled trial. Ann Intern Med 1999;130:7-13.
21. Harden PN, MacLeod MJ, Rodger RS, Baxter GM, Connell JM, Dominiczak AF, et al. Effect of renal-artery stenting on progression of renovascular renal failure. Lancet 1997;349:1133-6.
22. Ballardie FW, Roberts IS. Controlled prospective trial of prednisolone and cytotoxics in progressive IgA nephropathy. J Am Soc Nephrol 2002;13:142-8.
23. Ishida K, Ishida H, Narita M, Sairenchi T, Saito Y, Fukutomi H, et al. Factors affecting renal function in 119 985 adults over three years. Q J Med 2001;94:541-50.
24. Nakao T, Yoshino M, Matsumoto H, Okada T, Han M, Hidaka H, et al. Low-density lipoprotein apheresis retards the progression of hyperlipidemic overt diabetic nephropathy. Kidney Int Suppl 1999;71:S206-9.
25. Kubo M, Kiyohara Y, Kato I, Iwamoto H, Nakayama K, Hirakata H, et al. Effect of hyperinsulinemia on renal function in a general Japanese population: the Hisayama study. Kidney Int 1999;55: 2450-6.
26. Soma J, Saito T, Taguma Y, Chiba S, Sato H, Sugimura K, et al. High prevalence and adverse effect of hepatitis C virus infection in type II diabetic-related nephropathy. J Am Soc Nephrol 2000; 11:690-9.
27. Horio M, Orita Y, Manabe S, Sakata M, Fukunaga M. Formula and nomogram for predicting creatinine clearance from serum creatinine. Clin Exp Nephrol 1997;1:110-4.
28. Meunier-Durmort C, Poirier H, Niot I, Forest C, Besnard P. Up-regulation of the expression of the gene for liver fatty acid-binding protein by long-chain fatty acids. Biochem J. 1996; 319(Pt 2):483-7.
29. Lawrence JW, Kroll DJ, Eacho PI. Ligand-dependent interaction of hepatic fatty acid–binding protein with the nucleus. J Lipid Res 2000;41:1390-401.
30. Huang H, Starodub O, McIntosh A, Kier AB, Schroeder F. Liver fatty acid–binding protein targets fatty acids to the nucleus. Real time confocal and multiphoton fluorescence imaging in living cells. J Biol Chem. 2002;277:29139-51.
31. Wolfrum C, Borrmann CM, Borchers T, Spener F. Fatty acids and hypolipidemic drugs regulate peroxisome proliferator–activated receptors alpha- and gamma-mediated gene expression via

liver fatty acid binding protein: a signaling path to the nucleus. Proc Natl Acad Sci U S A 2001;98:2323-8.
32. Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 1990;347:645-50.
33. Wolfrum C, Ellinghaus P, Fobker M, Seedorf U, Assmann G, Borchers T, et al. Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein. J Lipid Res 1999;40:708-14.
34. Sheridan AM, Schwartz JH, Kroshian VM, Tercyak AM, Laraia J, Masino S, et al. Renal mouse proximal tubular cells are more susceptible than MDCK cells to chemical anoxia. Am J Physiol 1993;265:F342-50.
35. Humes HD, Nguyen VD, Cieslinski DA, Messana JM. The role of free fatty acids in hypoxia-induced injury to renal proximal tubule cells. Am J Physiol 1989;256:F688-96.
36. Matthys E, Patel Y, Kreisberg J, Stewart JH, Venkatachalam M. Lipid alterations induced by renal ischemia: pathogenic factor in membrane damage. Kidney Int 1984;26:153-61.
37. Ramsammy LS, Josepovitz C, Lane BP, Kaloyanides GJ. Polyaspartic acid protects against gentamicin nephrotoxicity in the rat. J Pharmacol Exp Ther 1989;250:149-53.